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# Metagenomic analysis of gut microbial communities from a Central Asian population

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## ABSTRACT

**Objective** Changes in the gut microbiota are increasingly recognised to be involved in many diseases. This ecosystem is known to be shaped by many factors, including climate, geography, host nutrition, lifestyle and medication. Thus, knowledge of varying populations with different habits is important for a better understanding of the microbiome.

**Design** We therefore conducted a metagenomic analysis of intestinal microbiota from Kazakh donors, recruiting 84 subjects, including male and female healthy subjects and metabolic syndrome (MetS) patients aged 25–75 years, from the Kazakh administrative centre, Astana. We characterise and describe these microbiomes, the first deep-sequencing cohort from Central Asia, in comparison with a global dataset (832 individuals from five countries on three continents), and explore correlations between microbiota, clinical and laboratory parameters as well as with nutritional data from Food Frequency Questionnaires.

**Results** We observe that Kazakh microbiomes are relatively different from both European and East Asian counterparts, though similar to other Central Asian microbiomes, with the most striking difference being significantly more samples falling within the *Prevotella*-rich enterotype, potentially reflecting regional diet and lifestyle. We show that this enterotype designation remains stable within an individual over time in 82% of cases. We further observe gut microbiome features that distinguish MetS patients from controls (eg, significantly reduced Firmicutes to Bacteroidetes ratio, *Bifidobacteria* and *Subdoligranulum*, alongside increased *Prevotella*), though these overlap little with previously published reports and thus may reflect idiosyncrasies of the present cohort.

**Conclusion** Taken together, this exploratory study describes gut microbiome data from an understudied population, providing a starting point for further comparative work on biogeography and research on widespread diseases.

**Trial registration number** ISRCTN37346212; Post-results.

## Strengths and limitations of this study

- These are the first high-resolution data on the gut microbiome of a Central Asian population. We show that these microbiomes are similar to those elsewhere while still exhibiting regional idiosyncrasy, including with regards to locally unique gene variants.
- Kazakh samples are significantly and strongly skewed towards a *Prevotella*-rich enterotype, a skew that holds for both autumn and winter samples, both MetS cases and controls, and both placebo and synbiotic study subjects.
- We are able to demonstrate significant associations between dietary factors and the microbiome in a large cohort quantified at high resolution.
- Participants are all volunteers from the capital city of Astana, all governmental employees and predominantly female. As such, they are not a representative sample of the Kazakh population as a whole in all regards.
- Due to probiotic genomes not having been sequenced, we cannot yet trace carriage of these strains at high resolution, and as such, cannot evidence that the effect of the synbiotic occurs via microbiome changes as opposed to direct or indirect effects on the host.

## INTRODUCTION

Microbial contributions to human health, as currently understood, involve digestion, metabolism of endogenous and exogenous compounds, modulation of immune defense mechanisms and hindering colonisation of the gastrointestinal tract by (competitor) pathogenic microorganisms.[1] Microbial cells produce many of the necessary enzymes for digesting carbohydrates and proteins in the colon, which the human host cells cannot.[2] Diet has strong influences on microbial composition and diversity,[3 4] alongside factors such as climate and geography surrounding the human host[5] or the genetics of that host.

All these factors could potentially affect the pathogenesis and course of various diseases, such as the metabolic syndrome (MetS), characterised by obesity, hypertension, high blood glucose and high levels of hard digestible fatty acids in the blood.[6] MetS is further strongly comorbid with more severe metabolic and cardiovascular diseases and is growing in prevalence worldwide.[7] According to epidemiological studies from 2016, the overall prevalence of type 2 diabetes (T2D) in Kazakhstan is 12.5%,[8] a number that is increasing over time.[8]

Thus, an understanding of the gut microbiome and its role in aetiopathogenesis becomes crucial, with a perspective towards personal diet control to more efficiently improve health.[4] Moreover, recognising that the extent of global variability in gut microbiome composition and reactivity remains unknown,[5] particularly in populations where traditional lifestyle practices may play a strong role, we here present the first metagenomic analysis of gut microbiota from Kazakh individuals, recruited from the administrative centre of Astana, comprising 84 male or female healthy subjects and MetS patients aged between 29 years and 75 years. We analyse these data in a global context and evaluate how the Kazakh microbiomes correlate with clinical and lifestyle parameters as well as the influence of the NAR synbiotic, derived from traditional Kazakh medicinal foods, on the gut microbiome. Thus, in addition to describing an understudied population, we also aimed to provide further knowledge into a pathology common to this population[8] and into an intervention that could possibly contribute to its treatment.

## METHODS

### Cohort

Eighty-four healthy and non-healthy male and female individuals aged 25–75 years were recruited in Astana, at the administrative centre of Kazakhstan. Ethnic structure is diverse and includes Kazakh, Russian, Tatars, Ukrainians, Uzbeks and Germans. The case group included participants with overweight, diagnosed diabetes and/or hypertension, thus presenting the MetS. Exclusion criteria included any evidence of taking antibiotics for 3 months or less prior to sampling. The consent documents were signed by all participants before faecal sample collection. Before the start of the study, all patients were examined comprehensively, including clinical and laboratory examination and survey. Clinical and laboratory data collected include anthropometrics, cardiovascular status (systolic and diastolic blood pressure and heart rate), blood lipid profile, levels of circulating inflammatory markers, immunologic status and the results of general inspection analysis of faeces (coprogram), as well as records of stool consistency and frequency, together with a questionnaire about habitual food consumption along with a consent form. The questionnaire included an assessment of the patient's health, familial anamnesis, standard metadata as per the MyMicrobes protocol (http://my.microbes.eu/) and a Food Frequency Questionnaire (FFQ) section. All physical measurements were made by a trained medical professional and include height, weight, waist and hip circumference, blood pressure and heart rate.

### Patient and public involvement

Participants were recruited from attendees of the Medical Centre Hospital of President's Affairs Administration of the Republic of Kazakhstan. Hospital employees conducted recruitment and instructed the participants both on the analysis and the correct procedure for collection and delivery of the stool sample and use of the synbiotic/placebo. Participants were not otherwise directly involved in either study design or participant recruitment. At the stage of recruiting, all patients received a complete set of information about the tested synbiotic, the research goals and all planned clinical and laboratory investigations. The patients independently and voluntarily decided to participate in the study. All patients were informed that they have the right to refuse further participation at any stage and confirmed their participation in the study by signing this informed consent. The results of the research will be disseminated to the public through publications in scientific journals and proceedings of profile conferences but not directly communicated to the individual participants beyond this. The patients were randomised to synbiotic or placebo using to the electronic database of the medical centre, without their health state playing any role in this randomisation. No evaluation of the effect of the intervention was communicated directly to the (anonymised for integrity purposes) participants.

### Sample collection

A stool sampling kit consisting of a sample collection tube, cotton swabs and sterile tissue papers was given to each subject. Human faecal samples were collected and frozen immediately. The collection procedure was repeated again with an average interval between samplings of 90±5 days. Faecal samples collected were placed at −20°C immediately after they were produced and at −80°C within 24 hours. All samples were maintained at −80°C until they were used for metagenomic studies.

### Sample processing and sequencing

#### DNA isolation from faecal samples

Total DNA was extracted from all faecal samples (two samples/individual) using an adapted G'NOME kit (BIO 101) protocol as described in Zeller *et al*.[9]

#### Library preparation and metagenomic sequencing

Samples were sequenced at the EMBL GeneCore facility using an Illumina HiSeq 2500. On average 2.7±1.1 Gbp of 100 base pairs (bp) paired-end shotgun sequencing reads was generated for each sample.

### Data processing

Reads were processed using the MOCAT pipeline[10] to determine bacterial species abundance under the metagenomic Operational Taxonomic Unit (mOTU) framework[11] and mapping reads to a previously described gut

microbial gene catalogue[12] in order to assess functional capacity. Furthermore, community ecological indices, including taxonomic richness and evenness as well as Shannon diversity, were determined based on rarefaction analysis of the mOTU data (following the procedure in Qin *et al*).[13]

### Data analysis

Computer analysis thus provides the following results: taxonomic composition of samples with respect to metagenome-derived (reference-free) taxonomic units (mOTUs[11]); taxonomic composition of samples with respect to a reference database of known microbial genomes[14] median abundance across each such gene group here used as a measure of abundance of each metagenomic species; taxonomic distance between samples (Bray-Curtis and log-transformed Euclidean distances between mOTU profiles); ecological diversity of samples (derived from subsampling of mOTU abundances); gene richness of samples (derived from subsampling hits to the 10M reference gene catalogue previously published[12]; enterotypes of samples (derived clustering together with samples used originally to identify enterotypes[15]; functional profiles of samples with respect to Kyoto Encyclopedia of Genes and Genomes (KEGG) modules and pathways (based on mapping of reads to the 263 sample reference gene catalogue previously described, annotated based on homology to KEGG annotated genes[16]; and functional profiles of samples with respect to antibiotic resistances (based on mapping of reads to the 10M gene catalogue previously described, annotated based on homology to antibiotic resistance genes in the ResFams database.[17]

### Data availability

Samples have been deposited to the ENA archive under accession PRJEB17632.

## RESULTS

### Cohort description and data collection

In total, 84 participants were enrolled in the study in 2015. These involved voluntary participants from two categories: one group diagnosed with MetS (n=58), and a second group of healthy controls (n=26). Antibiotic use in the last 3 months was an exclusion criterion. Stool samples were collected twice: once in summer (August) and once in winter (January) (168 samples in total). The current setup thus provides multiple dimensions of potential contrasts: summer/winter (with corresponding dietary changes), MetS patients versus healthy subjects and dietary effects. Additionally, all participants following autumn sample collection began a minor diet change: taking daily either synbiotic (ie, combining prebiotic and probiotic components) yoghurt or placebo as part of the study. The placebo was an inactive milk fermentation, whereas the synbiotic contains six probiotic strains,

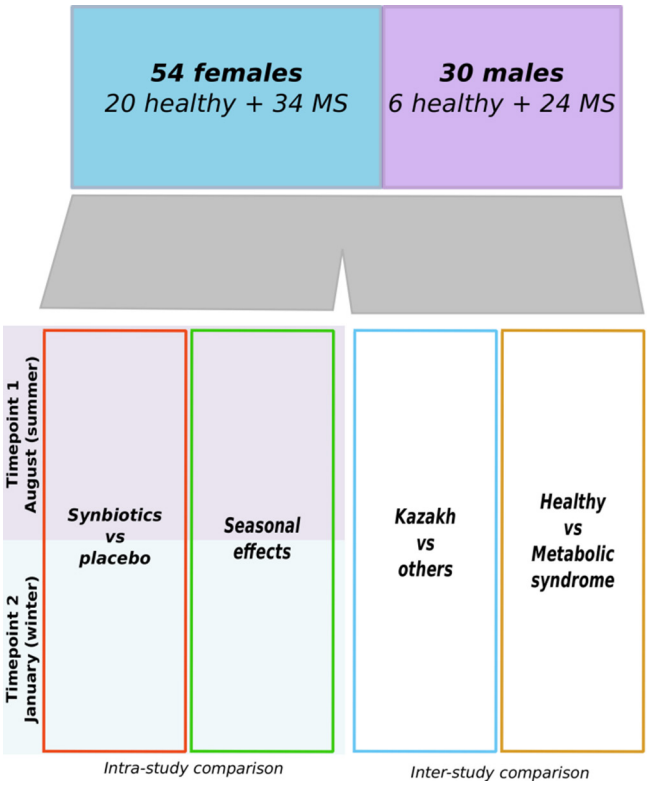


**Figure 1** Study design. This scheme shows the design of the study and the setup of the cohort. Eighty-four subjects (healthy or with MetS) were sampled twice, in summer and in winter, with half receiving placebo and half the NAR synbiotic. This setup allows multiple contrasts: seasonal variation, MetS cases versus control and differential effects of placebo versus synbiotics. In addition, Kazakh samples were placed into the a global context by comparison with other samples. MetS, metabolic syndrome.

as well as the prebiotics fish collagen and pectin (*data not shown*). Figure 1 highlights the design of the study.

The cohort by design is relatively homogeneous for lifestyle and socioeconomic status, as recruitment of participants was carried out in an Astana city hospital, which specifically treats employees of governmental organisations. The majority of participants moved to Astana within the last two decades, in the course of its establishment as an administrative centre. An overview of basic demographic data for the 84 participants is provided in table 1. Participants' ages ranged between 29 years and 75 years, with an average of 50.39 years (median 50 years). A slight majority of participants (54/84) were female. Additional data collected includes anamnesis of diseases other than the MetS, as well as anamnesis of family morbidity (specifically, morbidity of siblings or parents). To assess whether there is structure in these disease histories, we carried out a hierarchical clustering (Ward clustering on a binary distance measure) on these variables, resolving two major clusters of comorbidity visible in the cohort, as seen in online supplementary figure 1. Under this measure, most familial comorbidities cluster together with MetS status and variables denoting severity of any disease sufficient to limit daily activity, whereas most other diagnoses present

Table 1 Overview of demographic data of participants

| | Number of participants | Average age (years) | Sex (male/female) | BMI autumn (male) (kg/m$^2$) | BMI winter (male) (kg/m$^2$) | BMI autumn (female) (kg/m$^2$) | BMI winter (female) (kg/m$^2$) |
|---|---|---|---|---|---|---|---|
| Total | 84 | 50.39 | 30/54 | 27.9 | 27.8 | 26.8 | 26.7 |
| Subjects receiving NAR: yoghurt containing six probiotic species, collagen, pectin and inulin | 43 | 50.02 | 16/27 | 27.9 | 27.8 | 26.8 | 26.4 |
| Subjects receiving controls: yoghurt without supplements | 41 | 50.78 | 14/27 | 28.4 | 28.7 | 27.37 | 27.7 |
| Subjects with MetS | 58 | 49.03 | 24/34 | 29.1 | 29 | 29.1 | 29 |
| Healthy controls | 26 | 52.83 | 6/20 | 23 | 23.1 | 22.9 | 22.9 |

The table shows the basic study design. The 84 participants were sampled twice each for 168 samples in total. Of these, roughly half underwent synbiotic intervention with NAR. In parallel, roughly one-third of the study participants were metabolically healthy, with the remainder diagnosed with MetS. The table further shows basic demographics (age and sex) and BMI distribution at the different time points.
BMI, body mass index; MetS, metabolic syndrome.

in the cohort are scattered outside this cluster. Furthermore, 55% of MetS patients have anamnesis of T2D, hypertension, myocardial infarction or stroke in either parents or siblings, consistent with shared heredity of T2D with cardiovascular disease.[18]

### The composition of the gut microbiota of individuals from Kazakhstan

Comparing the composition of the Kazakh samples to that of other available datasets (US Human Microbiome Project data[19]), Spanish and Danish MetaHIT samples,[20] Swedish[21] and Chinese[13] T2D samples and controls, an initial view (PCoA breakdown of Bray-Curtis distances between samples in mOTU taxonomic composition space; online supplementary figure 2) reveals the Kazakh samples as clearly separable from European samples and from previously sequenced non-European microbiome datasets. While batch effects are a possibility in metagenomic analysis, the protocol of the present study was also used for the largest groups of European control samples (MetaHIT), suggesting these differences cannot be reduced solely to such artefacts.

Considering distinguishing taxonomic features of the Kazakh samples, many bacterial taxa are significantly enriched or depleted in this dataset (figure 2). Of phyla showing significant (Mann-Whitney U test False Discovery Rate (Benjamini-Hochberg adjustment), MWU FDR <0.05) differences in abundance between Kazakh and other datasets, Actinobacteria, Proteobacteria, Firmicutes and Bacteroidetes were found in all Kazakh samples, with several others also commonly found (online supplementary table 1). At the genus level, abundance of *Blautia*, *Bifidobacterium*, *Ruminococcus*, *Bacteroides*, *Eubacterium*, *Faecalibacterium*, *Prevotella*, *Streptococcus* and *Clostridium* all exceeded 1% in all samples. This is consistent with results obtained in an earlier study where a composite analysis using 16S amplicon sequencing conventional microbiological techniques was undertaken of the gut microbiota of Kazakh women.[22]

Previously, a metagenomic reference gene catalogue generated from 263 human gut samples was described.[23] Metagenomic reads from the stool samples in this study were mapped to this catalogue using the same procedure as there. Roughly 10% fewer reads on average can be mapped for each Kazakh sample (data not shown) than was the case for previously published sample sets. This suggests the possibility that the microbial diversity in Kazakh metagenomes is underexplored.

Analysing the composition of the gut microbiota of this cohort under the mOTU framework, we identify 22 such mOTUs (corresponding to species) that are core to the Kazakh microbiota in the sense that each were found in at least 90% of samples. These core operational taxonomic units (OTUs) primarily belonged to the genera *Faecalibacterium*, *Bacteroides*, *Dorea*, *Collinsella*, *Oscillibacter*, *Ruminococcus*, *Subdoligranulum*, *Coprococcus* and *Prevotella*.

### Enterotype analysis

Projecting the Kazakh samples into enterotype[15] component space (showing also 278 Danish MetaHIT samples for comparison) reveals Kazakh samples to be significantly (Fisher's exact test p<5e-14) and strongly (OR 7.24, 95% CI 4.09 to 13.12) skewed towards enterotype 2 (*Prevotella*-rich), a skew that holds for both autumn and winter samples, both MetS cases and controls, and both placebo and synbiotic study subjects (figure 3, table 2). Seventy-one per cent of Kazakh samples belong to this enterotype. It should be noted that while the present cohort consists of both healthy controls and MetS subjects, the same rough distribution of cases to control also hold for the MetaHIT Danish cohort, which we show for comparison, suggesting this enterotype shift towards *Prevotella* is idiosyncratic to the Kazakh population rather than any feature of metabolic disease.

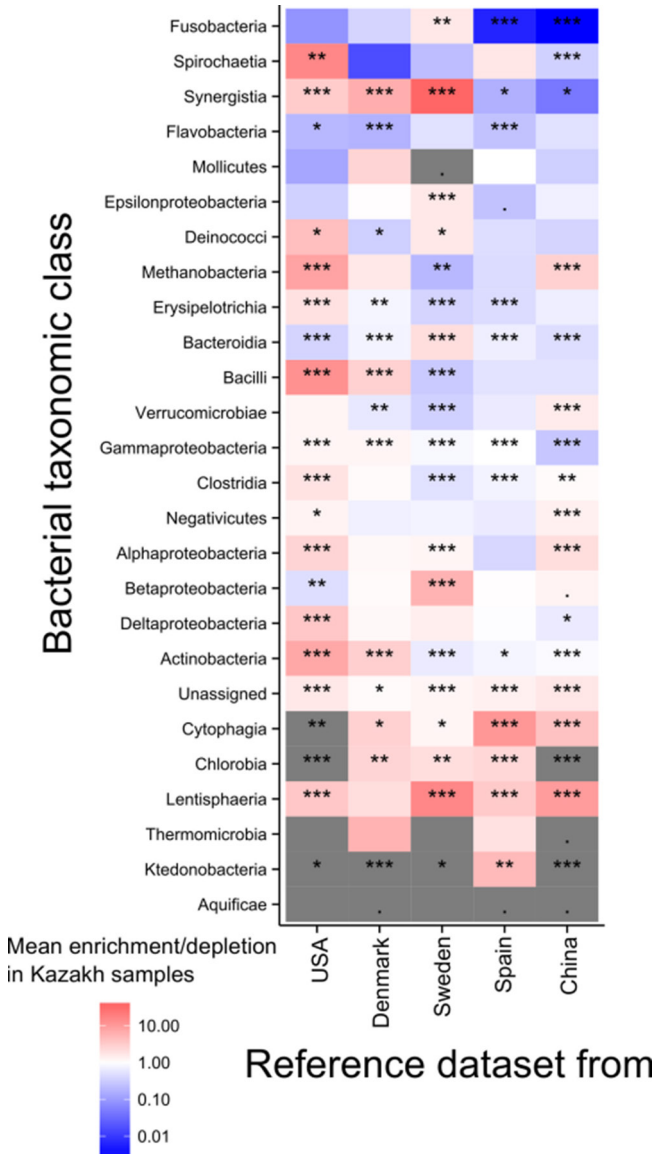


**Figure 2** Bacterial families significantly different in Kazakh metagenomes Heatmap view of significantly enriched/depleted bacterial families in the Kazakh metagenomes compared with those from reference datasets. Each column represents a comparison of the Kazakh data with each other dataset, and each row represents one bacterial family where at least one country comparison was significant. Colour scale shows the degree of change, as the ratio of mean abundance across the datasets. Asterisk markers denote statistical significance (Benjamini-Hochberg (BH) FDR scores from MWU tests comparing abundances. FDR <0.1; *FDR <0.05; **FDR <0.01; ***FDR <0.001). FDR, False Discovery Rate; MWU, Mann-Whitney U test.

Comparing autumn and winter samples, enterotypes remain stable over time; samples from the same donor are significantly (permutation test p<3e-5) more likely to remain stable over time than would be expected under a null model. Thus, whatever the mechanism behind the enrichment of *Prevotella*-type gut microbiomes in the Kazakh cohort, it is unlikely to reflect seasonal dietary or lifestyle changes.

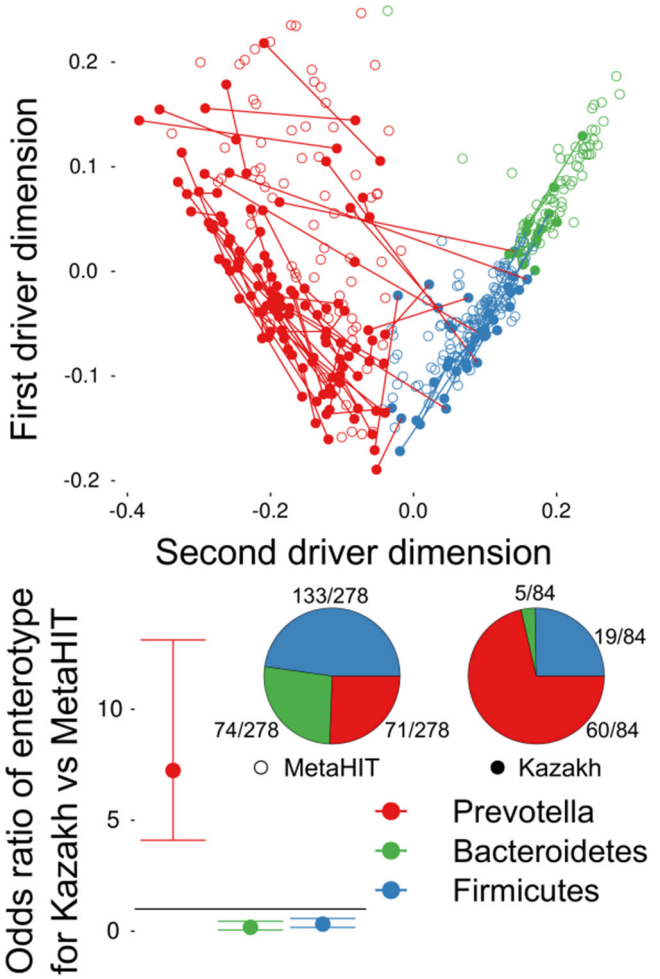


**Figure 3** Enterotyping analysis Kazakh samples fall mostly within the *Prevotella*-rich enterotype 2, compared with Danish MetaHIT samples. Scatterplot shows samples projected onto the first two driver dimensions of enterotype space, with solid markers for the novel Kazakh samples and hollow markers for 278 Danish MetaHIT samples. Colours signify enterotype clusters. Same-donor samples in autumn and winter are connected by lines. Error bar diagram shows 95% CI of OR for falling within each enterotype if samples are Kazakh rather than MetaHIT; the Kazakh samples are depleted for enterotypes 1 and 3 and enriched for enterotype 2. Pie charts show sample distribution across enterotypes in the two cohorts.

**Table 2** Comparative analysis of the enterotype distribution

| Enterotype | Kazakh (%) | MetaHIT (%) | Kazakh (N) | MetaHIT (N) |
|---|---|---|---|---|
| Bacteroidetes-rich | 6 | 27 | 5 | 74 |
| Prevotella-rich | 71 | 26 | 60 | 71 |
| Firmicutes-rich | 23 | 48 | 19 | 133 |

The table shows enterotype assignment counts for the novel Kazakh samples as well as Danish MetaHIT samples for comparisons, shown as sample counts (N) and as percentages (%).

### Gut microbiome correlates to MetS in the Kazakh cohort

Comparing MetS case samples to healthy controls reveals borderline significantly (MWU p<0.1) decreased

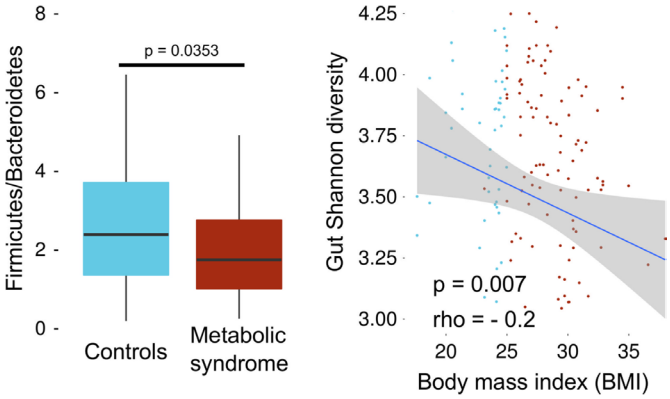


**Figure 4** The ratio of Firmicutes to Bacteroidetes (F/B) boxplot showing for the MetS and control samples the ratio of bacterial phyla *Firmicutes* to *Bacteroidetes*. This ratio is slightly but significantly (MWU p<0.0353) reduced in MetS cases compared with controls. MetS case samples also have significantly (MWU p<0.1) lower Shannon diversity and community evenness than control samples. MetS, metabolic syndrome; MWU, Mann-Whitney U test.

Shannon taxonomic diversity and community evenness between MetS cases and controls (figure 4). Across participants, the ratio of the bacterial phyla Firmicutes to Bacteroidetes (F/B ratio) ranges from 0.2 to 21. Several studies (see refs 24–26) have shown that such high ratios are characteristic of healthy young adults and that they decrease with age. This trend was not seen in the present cohort. However, the F/B ratio was significantly reduced (MWU p=0.0353) in MetS samples than in healthy controls. Previous studies (ref 27) have reported higher F/B ratios in obese subjects; these divergent accounts would be reconciled if such a ratio reflects an obesigenic habitual diet rather than obesity itself, as the MetS patients in the present study also have lower nutrient intake than controls, likely reflecting compliance with advice from their physicians following diagnosis.

Further analysis of MetS case samples compared with healthy controls reveals significantly (MWU test, BH FDR <0.1) different abundances of some bacterial species as well as some bacterial gene functional modules. Most significant differences concern relatively poorly characterised mOTUs, with the MetS microbiomes depleted of *Bifidobacteria* and some butyrate producers like *Subdoligranulum*, while enriched for *Prevotella*. For bacterial gene functional annotation the KEGG database was used.[28] Forty-four KEGG pathways exhibited significant differences in abundance between MetS and healthy control samples. Notably, MetS cases in this cohort are enriched in genes for lipopolysaccharide biosynthesis and depleted of various systems for transporting or using sugars. These functional and taxonomic changes are intercorrelated (figure 5) and may, again, either represent the disease pathology or else steps such as diet changes in order to treat it.

Since the definition of MetS relies in part on obesity, body mass index (BMI) of donors may arguably act as a confounding factor, in case such features are dependent on BMI. To test this scenario, this contrast test was repeated, instead checking significance with respect to whether a general linear model of each tested feature considering both MetS status and BMI performed better than one considering BMI only as a dependent variable in modelling abundance of each tested feature. No feature is significantly different in abundance between MetS and control samples under this measure, suggesting it is difficult to disentangle features unique to MetS from those distinct to obesity itself. Comparison of the features found to distinguish MetS cases from controls in the Kazakh samples are not found to distinguish MetS cases from controls in the MetaHIT cohort,[20] suggesting it is likely that health and lifestyle factors, as well as severity of the phenotype, here confounds any true metagenomic signature of the MetS.

## Concordance of diet and the gut microbiota

We detected no significant changes in the microbiome under synbiotic treatment compared with placebo, though treatment did improve clinical phenotype of MetS patients significantly (*data not shown*). An overview of clinical and laboratory data for the 84 participants is provided in table 3. Full medical data, including antibiotic use history, were also recorded, and diet at enrolment was assessed via FFQs. As participants completed FFQs at enrolment, we investigated whether gut microbial composition (at either time point) could be explained in part from this data. Absolute nutrient amounts per day were projected from the FFQ data, and the resulting profiles were correlated against gut abundance of different microbial taxa. Few or no association were found at broader taxonomic levels, whereas at the level of microbial taxa (mOTUs), 17 (11 not yet well characterised) had significant (Spearman FDR <0.05) associations to one or more nutrient categories. Because of the dense structure of the resulting network, we visualise it (figure 6) as a power graph[29] wherein nodes are grouped together based on their shared relationship to other sets of nodes.

Most associations were negative, signifying how higher intake of of some foods is associated with reduced abundance of some bacteria.

Seen as a whole, these data suggest that multiple poorly characterised species, particularly Prevotellas, Firmicutes and Clostridiales, are reduced in abundance either from overall higher food intake, or specifically intake of fats and sugars. Only two associations were positive, namely those between the alcohol consumption cluster (M) and a *Bifidobacterium* mapped to *B. catenulatum* and *B. pseudocatenulatum*. While our data do not allow verification, one possible explanation is that many popular Kazakh alcoholic beverages are based on milk fermentations (eg, koumiss; see ref 30).

## Seasonal changes to the gut microbiota

No tested single microbiome feature was significantly different in gut abundance between corresponding summer and winter samples (paired MWU test, requiring

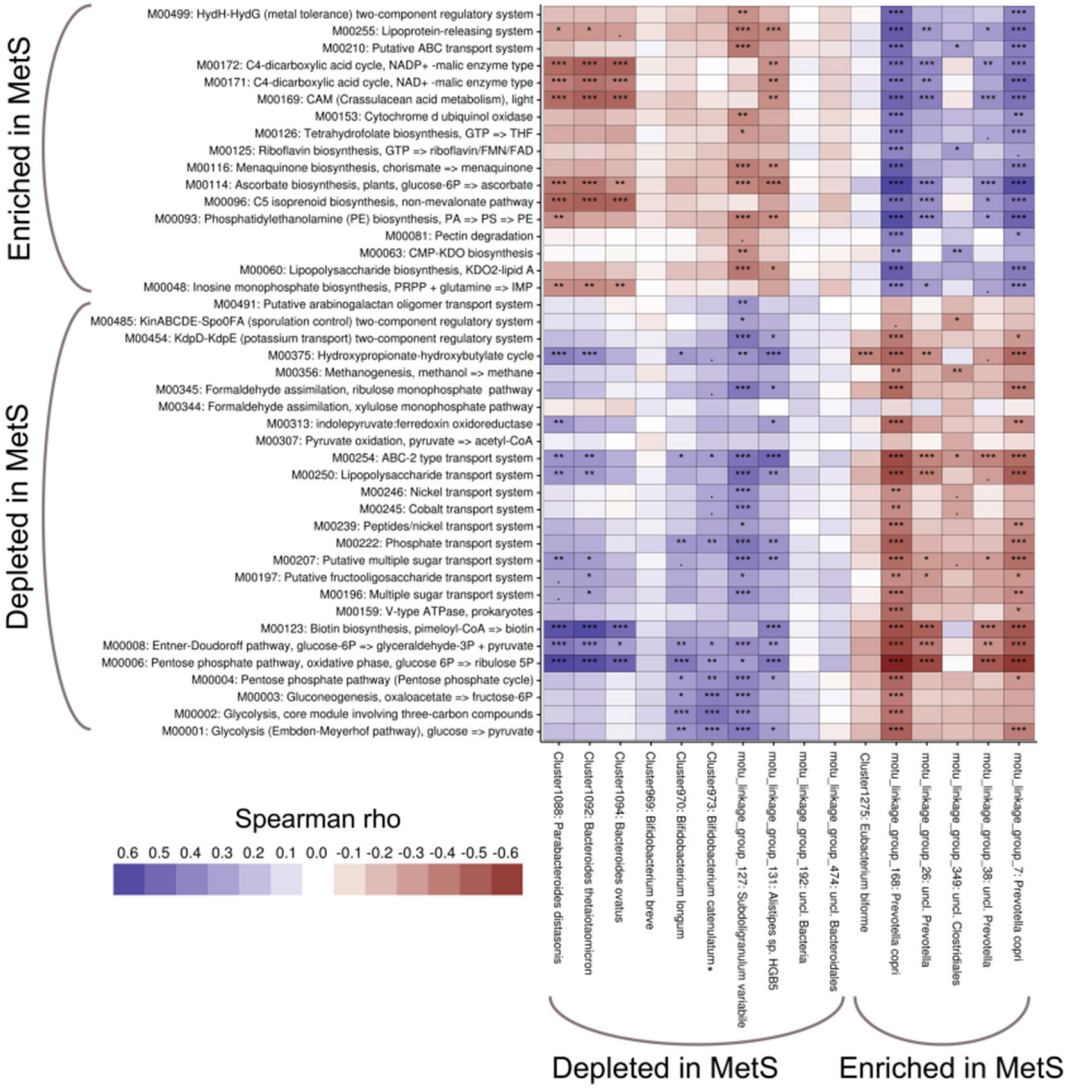


**Figure 5** A heatmap view of the mOTU and KEGG module MetS signature Heatmap view of significantly enriched/depleted bacterial taxa (mOTUs) and functional modules (KEGG) in Kazakh MetS samples compared with controls. Shown are all such taxa (horizontal axis) or modules (vertical axis) significantly (MWU FDR <0.1) different between cases and controls, along with their Spearman rho correlations, with significant (Spearman FDR <0.1) correlations marked through asterisk labels (+:p<0.1, *p<0.05, **p<0.01, ***p<0.001). FDR, False Discovery Rate; KEGG, Kyoto Encyclopedia of Genes and Genomes; MetS, metabolic syndrome; mOTU, metagenomic Operational Taxonomic Unit; MWU, Mann-Whitney U test.

BH FDR <0.1 for significance). However, a multivariate analysis (modelling Bray-Curtis intersample distances from sample metadata concordances, table 4, online supplementary figure 3) shows a significant effect of season (analysis of variance (ANOVA) FDR <0.008) as well as of MetS status (ANOVA FDR <6.3e-8), though not of synbiotic use.

### Analysis of the Kazakh gut antibiotic resistome

During 2014, 88.2 million packages of antibiotics were sold in Kazakhstan, with an increase of 33.7% during that year. This intensive use suggests a potential risk of antibiotic resistance development in human-associated microbiomes. However, in the present study, overall antibiotic resistance potential[23] of gut microbes was not significantly higher in Kazakh than European samples. Figure 7 shows an integrated view of the antibiotic resistome for Kazakh samples, separated into MetS case samples and controls, compared with those from other datasets. In brief, the relative abundance of known antibiotic resistance genes is comparatively low in the Kazakh samples (figure 7, left panel). However, the relative proportion of each gut community that consists of taxa, which are known to potentially carry resistance genes, is relatively high (figure 7, right panel).

## DISCUSSION

Studies of the gut metagenome is a promising field for personalised medicine and may provide a unique tool for treatment of a range of recalcitrant diseases. Since 2005, several studies have been published characterising gut metagenomes from host populations in countries in

Table 3 Overview of clinical and laboratory data of participants

| Variables | MetS | | Healthy | |
|---|---|---|---|---|
| Number of samples | Man, n=24 | Woman, n=34 | Man, n=6 | Woman, n=20 |
| BMI (kg/m$^2$) | 29.0±2.9 | 29.1±3.1 | 23.0±1.6 | 22.9±2.2 |
| Waist circumference (cm) | 99.5±8.4 | 91.2±11.2 | 89.5±15.6 | 76.8±9.7 |
| Hip circumference (cm) | 116.4±24.8 | 110.4±9.4 | 104.9±6.2 | 100.2±10.9 |
| Stool frequency (ordinal scale, see legend) | 1.81±0.4 | 1.97±0.5 | 2.0±0.6 | 2.15±0.5 |
| Stool consistency (Bristol scale value) | 3.43±1.2 | 3.05±1.2 | 4.4±1.5 | 4.08±0.5 |
| Haemoglobin (g/L) | 145±15.5 | 126±14.4 | 147.1±10.3 | 123±12.3 |
| Glucose in blood (mmol/L) | 5.4±0.5 | 5.25±0.9 | 5.8±0.8 | 4.9±0.3 |
| Triglyceride (mmol/L) | 1.82±1.03 | 1.42±0.69 | 1.26±0.5 | 0.96±0.2 |
| Cholesterol (mmol/L) | 4.89±0.9 | 5.12±0.9 | 5.1±1.6 | 4.5±0.9 |
| HDL (mmol/L) | 3.01±0.7 | 2.98±0.8 | 3.27±1.2 | 2.50±0.6 |
| LDL (mmol/L) | 0.98±0.2 | 1.30±0.5 | 1.24±0.4 | 1.39±0.5 |
| Systolic BP (mm Hg) | 125.6±15.9 | 126.7±20.7 | 118.3±13.2 | 107.7±10.3 |
| Diastolic BP (mm Hg) | 81.9±10.5 | 80.1±8.8 | 78.3±13.2 | 69.4±8.7 |
| CRP (mg/L) | 2.43±2.4 | 4.0±3.81 | 3.15±2.8 | 2.2±2.3 |
| Immunoregulatory Index (ratio) | 1.13±0.4 | 1.22±0.36 | 1.14±0.4 | 1.24±0.3 |

The table provides data of clinical and laboratory investigations (shown as mean±SD where defined) for all 84 participants at enrolment. Stool frequency is measured on an ordinal scale (1: 1–2 times/day, 2: daily, 3: every other day, 4: weekly, 5: less than weekly).
BMI, body mass index; BP, blood pressure; CRP, C reactive protein; HDL, high-density lipoprotein; LDL, low-density lipoprotein; MetS, metabolic syndrome.

Europe[31], South East Asia,[32–34] Africa[35] and the Americas,[36] under normal and pathological conditions. Such work has also identified new biomarkers of disease and suggested new approaches to diagnostics and therapy.[9 15 34 37–43] The present study represents the first deep-sequencing characterisation of the gut metagenome of a Central Asian population, drawn from samples from inhabitants of Kazakhstan. We observe that the distribution of enterotypes is strongly unlike that of other datasets, and we identify bacterial taxonomic groups significantly enriched and depleted in Kazakh individuals, with some features like elevated *Escherichia* also shared with Russian, Mongolian and East Asian populations, despite technical platform differences between these studies.

Comparing the Kazakh metagenomes with published 16S profiles of Mongolian gut microbiomes reveal both similar and distinctive features.[44] All these are likewise found among the core constituents we identified in the Kazakh gut microbiota, with the exception of *Subdoligranulum*, previously identified as a potentially protective factor against T2D.[45] Concerning East Asian microbiomes, studies have been made of the gut microbiomes of Koreans.[34] Comparing those results with the present Kazakh dataset reveals substantially different dominant bacterial taxa. Representatives of genera *Oscillibacter*, *Subdoligranulum* and *Fusobacterium* were found in the gut microbiomes of Kazakhs, but only sporadically, whereas they were ubiquitous in Korean samples (referring to the reported analysis in ref 46). The genus *Lachnospira*, however, was not identified in any of the Kazakh samples studied in the present work but was common in Korean samples. Notably, *Lachnospira* commonly persists in the gastrointestinal tracts of pigs. The transfer to the human gut of bacteria colonising that of food animals ('farm-to-fork') has been well documented.[47] Whereas pork is a common ingredient in Korean cooking,[46] it is largely absent from the Kazakh diet, which is also supported by the present FFQ data (data not shown).

Analysis of a Russian cohort using the SOLiD platform[37] revealed enterotype 1 (Bacteroidetes-rich) as rare in that population, and we here observe the same trend in Kazakh samples, suggesting common underlying factors. While broadly similar, the Russian microbiomes reported previously also differ from our present findings in the Kazakh microbiomes, as those exhibit relatively low *Lactobacillus* abundance whereas the Kazakh do not.

In both healthy and MetS Kazakh samples, we frequently see multiple opportunistic members of the microbiota. Inspection of coprograms revealed *Escherichia coli* as the most prevalent coliform found in these samples. Similar presence of high fractions of *E. coli* were also reported in previous studies of Russian (SOLiD sequencing, some donors from regions adjacent to Kazakhstan) and Mongolian (454 sequencing and qPCR) microbiomes,[37 44] and can further be seen enriched in Chinese compared with European samples.[45] It is possible that this high background level corresponds to some degree to antibiotic exposure, whether in medicine or food production, as *Escherichia* generally carry more antibiotic resistance genes than other members of the gut microbiota.[45]

Beyond characterisation of the gut metagenomes of healthy Kazakh individuals, we compared such samples

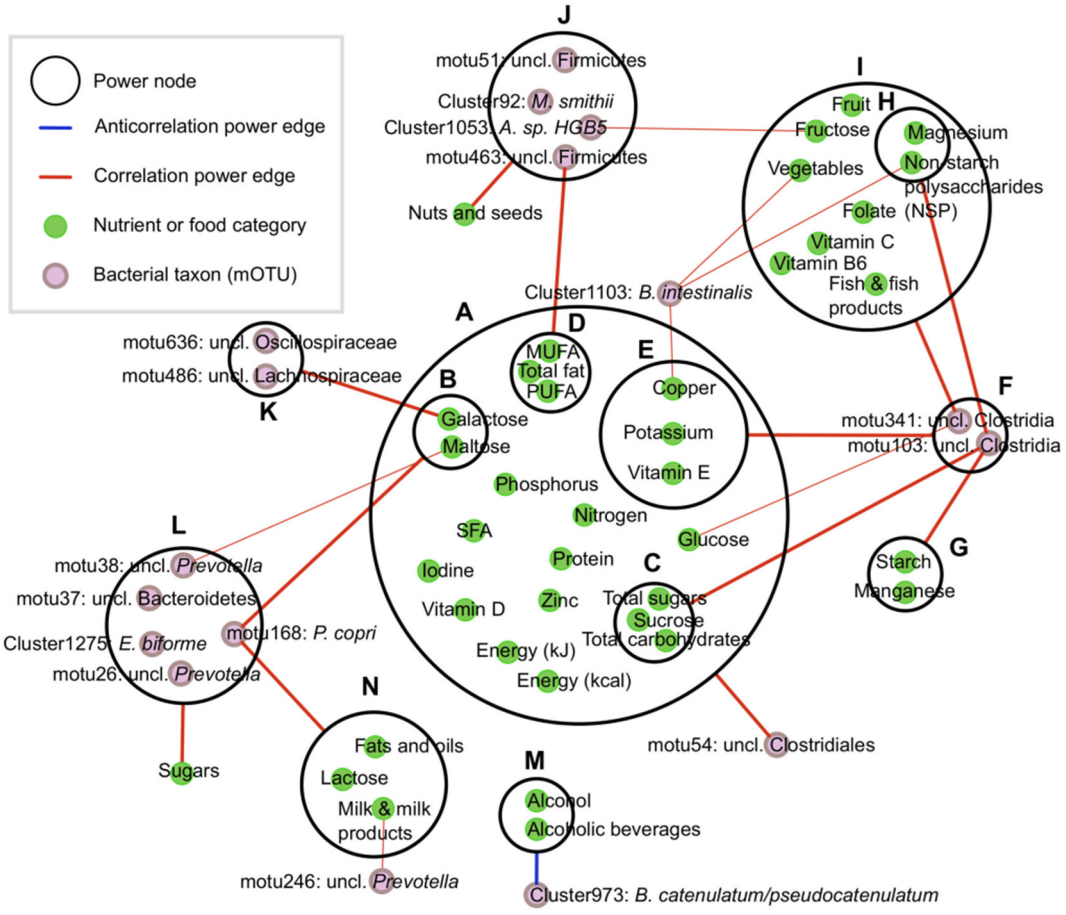


**Figure 6** Diet–microbiome associations in the Kazakh cohort power network visualisation of significant (Spearman test FDR <0.1) associations between dietary measurements and gut microbial composition (bacterial mOTU abundances). FDR, False Discovery Rate; mOTU, metagenomic Operational Taxonomic Unit. A central power node (A) containing subnodes for sugars (B and C) and fats (D) along with some minerals, as well as measures for overall energy consumption, likely represents total food intake, with an unclassified Clostridiales (family level assignment) being depleted as this measure rises. The mineral subcluster is anti-correlated with several unclassified Clostridia (class level assignment) (F), individually anticorrelated with clusters involving polysaccharides and minerals (G and H) and a cluster representing intake of fish, fruits and vegetables (I). Another cluster (J) of unclassified Firmicutes, the archaeon *Methanobrevibacter smithii* and an Alistipes, anticorrelated with consumption of nuts and seeds, with one unclassified Firmicute also anticorrelated to fat intake. Galactose intake was anticorrelated with abundance of two unclassified bacteria from families Oscillospiraceae and Lachnospiraceae (K), respectively. Overall sugar intake was anticorrelated with a cluster (L) involving several Prevotella, including *Prevotella* copri, *Eubacterium biforme* and an unclassified Bacteroidetes. *P. copri* show further anticorrelation with another sugar cluster (B) and a cluster containing fats, oils, lactose and milk products (N).

with those from MetS[48] patients. Unlike most other such studies where an increased F/B ratio was found associated with obesity and MetS, we found a significantly (though weakly) reduced F/B ratio in MetS participants in the present cohort compared with controls. Likewise, while we found significant gut microbial species associations to MetS status, these do not replicate in a European cohort[20] and also cannot be effectively disentangled from associations with overweight or lifestyle changes undertaken by the participants in response to their condition. Taken together, this underscores the MetS as a complex disease where possibly multiple different dietary patterns all could contribute while having different effects on the gut microbiome, suggesting significant risks of confounding in such studies unless very carefully controlled for.

The traditional diet of Kazakhstan is very different from either European or East Asian cuisine. Most Kazakh individuals have a high intake of red meat (especially horse), black and/or green tea (average 6–10 cups a day), fermented milk products and large amounts of butter-fried baked goods. We find distinct and significant effects of the diet of the study participants on the composition of their gut microbiomes, mostly on poorly characterised taxonomic groups. Further research on larger cohorts still, as well as thorough meta-analysis, will be required to fully chart these dependencies, including the extent to which they may underlie regional differences in microbiomes. If we can robustly understand dietary impact on the microbiome, we may become able to manipulate those ecosystems, and the health states they impact,

**Table 4** Taxonomic distance regression

**ANOVA of a linear model regressing Bray-Curtis intersample distances on donor, timepoint, disease status and treatment status concordance**

| | Df | Sum of square deviations | Fraction of variance explained, % | F-statistic | Pr(>F) |
|---|---|---|---|---|---|
| Same/different donor | 1 | 4.89 | 2.47 | 359.41 | <2e-16 |
| Same/different timepoint | 1 | 0 | 0.00 | 0.02 | 0.9 |
| Same/different MetS status | 1 | 2.59 | 1.31 | 190.37 | <2e-16 |
| Same/different synbiotic status | 1 | 0 | 0.00 | 0.2 | 0.65 |
| Residuals | 14023 | 190.81 | 96.23 | | |
| | Sum | 198.29 | 100.00 | | |

ANOVA of a linear model regressing Bray-Curtis intersample distances on donor, timepoint, disease status and treatment status concordance. Same-donor samples are significantly more similar to each other than to samples from other donors, reflecting individual-specific gut microbiota over time. Samples also are significantly more different if they are discordant for MetS status, whereas no such effect is found either for season of sampling or for placebo/synbiotic status.
ANOVA, analysis of variance; MetS, metabolic syndrome.

through dietary interventions.[4 7] The present study was in part intended to test the effects on the microbiome of on one hand seasonal change (resulting in shifts in environmental factors, time spent indoors, and summer vs winter diet), and on the other hand, the effect of a synbiotic treatment based on traditional milk fermentations combined with prebiotics.

While this synbiotic was significantly associated with protection from and even reversal of a seasonally associated increase in BMI common in Kazakhstan (*data not*

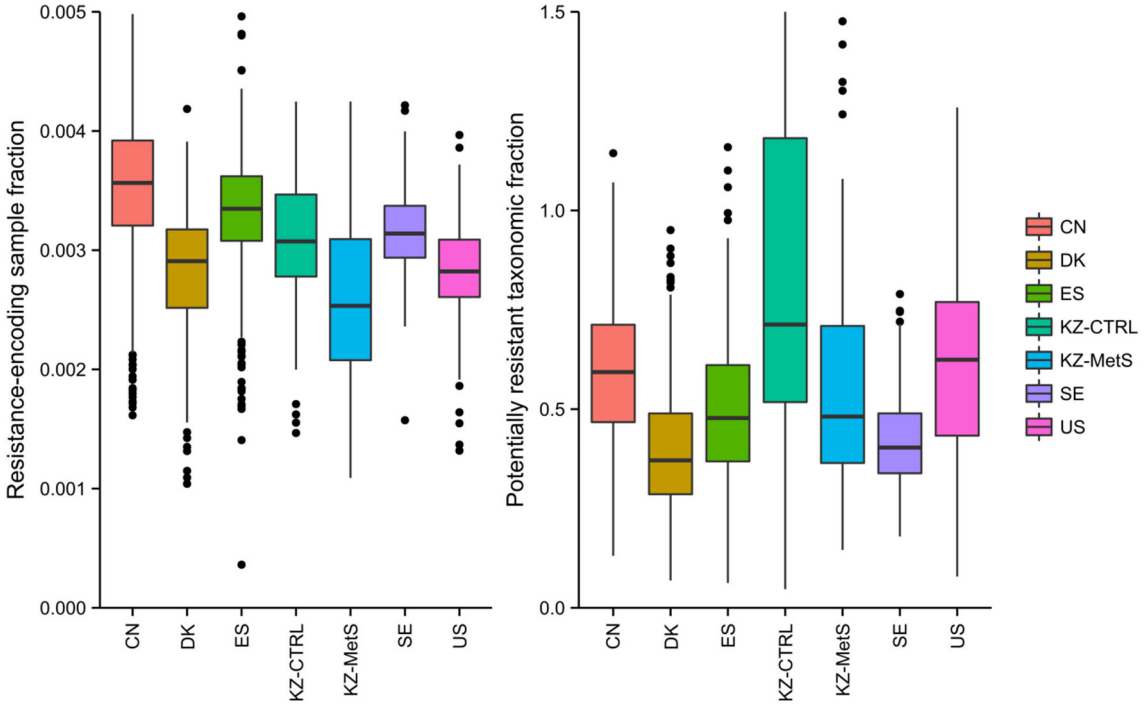


**Figure 7** Comparative gut resistome analysis of the Kazakh cohort. The left panel shows as boxplots the relative fraction of DNA from gut microbiome samples, which encode known antibiotic resistance genes. These fractions reflect both taxonomic composition of the samples and any enrichment, which may be in place for antibiotic resistance genes, such as selective pressure from exposure. The right panel shows what fraction of DNA from each sample derives from bacteria assigned to taxa (at the level of species clusters) where there are genomes known to contain antibiotic resistance genes. CN, China; DK, Denmark; ES, Spain; KZ-CTRL, Healthy Kazakh; KZ-MetS, Metabolic Syndrome Kazakh; SE, Swedish; US, United States of America.

*shown*), metagenomic analysis revealed neither seasonal differences nor any difference between participants receiving synbiotic or placebo. Previously, meta-analysis has shown similar results, in that the use of probiotics/synbiotics often does not lead to significant changes in diversity and richness of the gut microbiota.[47] Given this finding on one hand of a significant change in host phenotype under synbiotic treatment, and on the other hand, no significant microbiome compositional changes associated with this difference, further studies clearly are needed into mode of action of such synbiotics. It is conceivable that either very strain-specific properties of the probiotic component plays a part, or else that the prebiotic component affects the human host either directly or through effects on satiety and thus food consumption.

Concerning our observations of unexpectedly low extent of seasonal effects, Zhang *et al*[44] reported similar results from a study of gut microbiome variation over the year in Mongolian participants: while rural participants exhibited clear seasonal gut microbiome changes, reflecting shifts in diet, no such effects were found in subjects from urban areas, suggesting that the absence of a clear seasonal signal here likewise may reflect the urban lifestyle of the participants. This highlights how further studies into the microbiome of Kazakh individuals might aim to contrast rural and urban populations.

Concerning our findings of no higher overall resistance gene carriage in Kazakh samples compared with Western ones despite high sales of antibiotics, there are several ways to interpret these data. The Kazakh samples may as yet truly contain only low amounts of antibiotic resistance genes. Alternately, such genes may be present but sufficiently different from other such genes known and characterised elsewhere that they are not yet identified, suggesting functional metagenomic analysis for novel resistance genes may be fruitful. The relatively higher abundance of bacterial taxa, which can carry resistance genes, even if such genes were not found here, may suggest that composition broadly has been affected by antibiotic exposure, though further analysis would be required to formally test this.

Our study has a number of limitations. First, participants were all volunteers from the capital Astana, attached to the same hospital and occupying a similar social position. This is not a representative sample and may not reflect all persons across Kazakhstan and Central Asia more generally. Given that thus far genomes are unavailable for the probiotic strains used, it is possible that we fail to observe subtle metagenomic shifts involving carriage of these strains. Furthermore, the FFQs were filled only during participant enrolment, meaning that changes in diet during winter or following synbiotic treatment cannot be assessed directly. It is further possible that the study lacks statistical power to assess subtle changes in microbiomes more generally. Further research will be necessary to assess the impact of diet and environmental factors on the gut microbiota and its role in the development of lifestyle-related diseases, particularly as they may increase following the transition of societies from a traditional to a more modern lifestyle and diet.

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**Contributors** AK prepared the study protocol. ZK, VB and MU recruited participants. SK and TN interviewed participants. SK and TN collected biomaterials with input from AYV. ZK, VB and MU conducted clinical laboratory examinations. SK, SK-L, IL, AYV and TN handled sample logistics. SK and TN stratified participants by group. SK provided the tested synbiotic. AK and SKF analysed the results of clinical trials. AYV, MD, RH, VB and SK-L performed sample extractions and sequencing. IL, PIC and SS performed quality control and metagenomic sample computational processing. SKF conducted statistical and bioinformatic analyses. SKF and AK wrote the manuscript, with critical input from PIC, SS, IL and PB. ZZ and PB designed the study and supervised the work. All authors read and approved the final manuscript.

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